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Behaviour of Transplanted Tumours and Role of Matching in Rejection

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Abstract

Background: Tumour transfer/development is one of the more serious risks associated with transplantation. The behaviour of a tumour can be unpredictable in immunosuppressed recipients. We report a highly sensitive method to monitor tumour behaviour in real time in a rodent tumour transplant model. This paper also explores the effect of MHC matching on tumour growth among control and immunosuppressed hosts.

Methods: Luciferase expressing Wistar rat kidney tumour cells were transplanted into either Wistar or Lewis recipients which mimics a well and poorly matched combination to assess the effects of MHC matching on transplanted tumour cells. Experimental groups included controls with no immunosuppression and animals immunosuppressed with Cyclosporine. The latter group was further divided into a continuous treatment group which received four weeks of immunosuppression and a treatment withdrawal group where immunosuppression was stopped after two weeks to assess the effects of rejection on tumour growth.

Results: All the tumour cells were rejected in the control animals that received no immunosuppression, within 2 weeks among well-matched combination and within one week in the poorly matched combination ($p < 0.001$). The transplanted tumour cells continued to grow in both well-matched and poorly matched groups who were treated with cyclosporine, but growth was significantly faster in the well-matched combination ($p < 0.033$). After treatment withdrawal the tumour cells were rejected in all the animals of the poorly matched group compared to 50% in well matched animals within the four-week study period ($p < 0.039$).

Conclusion: In the absence of immunosuppression the hosts reject the transplanted tumour cells, and the anti-tumour response is stronger when there is a greater mismatch in MHC with the recipient. In the presence of Cyclosporine immunosuppression the tumour continues to grow, however, after withdrawal of the immunosuppression, tumour clearance is quicker in the poorly matched background. This data supports the idea of expansion of the donor pool by using kidneys after ex vivo resection of small renal tumours and that these organs should be transplanted into a less well-matched HLA recipient. We hypothesise that should a tumour recurrence occur a poorly matched recipient could clear the tumour through withdrawal of immunosuppression.

Word Count: 351

Key words:

Immunosuppression, Rejection, Renal, Tumour, Transplantation

1 **Background**

Transplantation has revolutionised the treatment of patients with renal failure. It not only improves quality of life but also has a significant survival advantage compared with dialysis(1). Although graft survival and the absolute number of allografts has increased over the past couple of decades, there remains a large gap between the number of organs available and potential recipients(2). Over the years new sources of organs have been explored but the problem persists and there is still a need to increase donor numbers.

There is a large body of evidence that patients with small renal cell carcinomas (RCC) can be treated with nephron sparing surgery (NSS) with comparable outcomes to the previous gold standard of radical nephrectomy(3,4). Consequently for a patient electing to have their whole kidney removed for a small RCC there is a potential for the removal of the tumour and then allotransplantation of the remaining kidney. This approach has been utilised by a few groups with good results(5–9). One of the most important and perhaps potentially dangerous differences between a urology patient that has undergone NSS for a small RCC and a potential allograft recipient of an NSS kidney is that transplant recipients are on lifelong immunosuppression. Immunosuppressive agents inhibit the natural checks on cancer cells by the immune system. It is not known how tumour cells will behave in a HLA incompatible immunosuppressed host, if there is any inadvertent transplantation along with such restored kidneys.

In the absence of any immunosuppression the allograft is rejected. Theoretically any tumour cells transplanted along with the allograft should be rejected as they both

originate from the same donor. However, cancerous cells have the ability to make themselves less immunogenic thereby evading the donor immune system in the first instance and it is not clear how they will behave in a new host (10).

2 Objectives

The aim of this study was to establish a rodent tumour transplant model and study the effects of immunosuppression on tumour growth. The other main aim was to study the effects of acute rejection on tumour cells in a transplantation setting.

3 Study Design and Methods

3.1 Cell culture

The tumour cell line, BP36b was acquired from Riken Bio Resource Centre (BRC) Cell Bank© Japan. This is a rat kidney tumour cell line derived from male Wistar rats that received N-ethyl-N-hydroxyethylnitrosamine (NHEN) in drinking water to induce tumour growth. The cell line is stable and maintained its characteristics after 100 passages over a 3 year period(11). Cells were grown in RPMI 1640 supplemented with glutamine and antibiotics (penicillin 10,000 units ml⁻¹, streptomycin 10 mg ml⁻¹, gentamicin 50 µg ml⁻¹ and amphotericin B 25µg ml⁻¹). The doubling time of the cell line was consistent with the reported time in the literature (17 hours)(11).

3.2 *Transfection*

For real time *in vivo* imaging of the tumour cells, the cell line was transfected with a commercial lentiviral construct that is stably integrated and constitutively expresses the enzyme luciferase for bioluminescence and Green Florescent Protein (GFP) for florescence(12,13). Puromycin ($10\ \mu\text{g ml}^{-1}$) was used for selection of stable transfectants.

Puromycin supplemented media was replaced every 48-72 hours to select for single colonies of stable transfectants. Transfectants were initially assessed by the expression of GFP by florescence microscopy. Bioluminescence was determined initially by a luminometer and then by direct visualisation using the IVIS® spectrum imaging system (Caliper Inc.) (Fig 1).

3.3 *Tumour transplantation*

Animals were injected with a fixed number of cells (1.8×10^7) into the right flank under Isoflurane anaesthesia after shaving the fur. The animals were anaesthetised in the induction chamber of the IVIS spectrum imaging system and then transferred into the dark chamber where they were scanned for varying lengths of time (60 -300 seconds). Animals were kept anaesthetised in the imaging chamber to enable long exposure times required to detect even very faint bioluminescent signals. Luciferin was injected intraperitoneally at the dose of 150mg/kg 10-15 minutes before scanning to allow circulatory distribution throughout the animal before detection. Timing of luciferin injection was calculated by plotting the kinetic curve prior to the experiments. To compensate for variations in Luciferin distribution, 2-3 images were

taken of each animal at different time points and the only the image with the strongest signal used for further analysis. Regions of interest (ROIs) were the areas of cell injection and any other areas with positive signals. The background luminescence was calculated for each animal and signal intensity was calculated by subtracting this from the ROI value to get the accurate value of signals from the transplanted tumour cells (Fig 2).

3.4 *Experimental groups*

To study the effects of matching on transplanted tumour growth two different strains of rats, Wistar and Lewis were used. Since tumour cells were of Wistar origin, when injected into Wistar rats (outbred)(14) this combination served as a well-matched group as both the animals were of the same strain. Despite the similarities between the tumour cell line and the recipients, these animals were not true syngeneic to the tumour cells due to being outbred(15). The other group was of inbred Lewis animals that served as a poorly matched group due to transplantation across the strain, leading to more marked immunological differences.

To keep the variables to a minimum, only male Wistar or Lewis rats were used for experiments as follows:

Controls; not receiving any immunosuppression and ***Cyclosporine (Cyc) group***; receiving 25mg/kg of Cyc daily via oral gavage. The cyclosporine group was further divided into **treatment continue group** receiving four weeks of continuous immunosuppression and the **treatment withdrawal group** where immunosuppression was stopped after 2 weeks to study the effects of rejection on the

transplanted tumour cells. All the animals were kept in a clean air conditioned rodent area with 12 hour dark/light cycle and were fed standard rodent blocks and with free access to tap water. Animals were weighed weekly to adjust the doses of Cyclosporine and Luciferin.

3.5 *Statistical analysis*

To detect a five-fold difference in tumour size with a standard deviation of 0.2 with a 90% certainty and alpha of 0.05 we calculated a sample size of 6 rats per group. Statistical analysis was performed with the PASW 18.0.0 (IBM Inc. 2009) and GraphPad prism (Version 5.04 GraphPad Inc.) softwares. The normality of the data was tested prior to performing either ANOVA or non-parametric Mann-Whitney tests accordingly.

4 Results

4.1 *Controls*

The kinetics of tumour rejection was first studied in the absence of immunosuppression. With *well-matched animals* there were still good signals at week one, but all the animals subsequently rejected the tumours cells and lost signal, even after long exposure, at week two. All *poorly matched animals* rejected the tumour cells and lost signal within the first week ($p < 0.001$ at week 1) (Fig 3).

4.2 Cyclosporine treatment

The effect of Cyclosporine on the rate of rejection was then studied in well-matched and poorly matched groups. These groups were further sub-divided into the animals receiving the immunosuppression for a full four weeks and the animals receiving the treatment for 2 weeks followed by treatment withdrawal. The rats in the treatment withdrawal group were scanned as normal for the study period of four weeks before euthanasia.

The tumour continued to grow in both the well and poorly matched animals when immunosuppressive *treatment was continuous*. There was no significant difference in the growth of the transplanted tumour cells in the initial three weeks of the study, however growth was significantly faster in the well matched Wistar animals compared to the poorly matched Lewis rats (**p 0.033**) by week 4 (Fig 4).

In 4 of 8 animals of the well-matched Wistar rats after *treatment withdrawal* tumour signal could still be detected at 2 weeks post treatment withdrawal. However, in the poorly matched Lewis animals the whole group had rejected the tumour by the end of the study period (two weeks post treatment withdrawal) (**p 0.039**) (Fig 5 & 6).

5 Discussion

Better immunosuppressive therapies have resulted in long allograft survival with reduced side effects. The risk of cancer development, however, even from standard allografts without any obvious donor malignancy still persists. The initial results of function and recurrence rates from transplanting restored organs after *ex vivo* resection of tumour remain favourable(16) from the limited data available so far.

However, there remain some serious questions regarding the safety of such an approach in immunocompromised hosts. The behaviour of a tumour in a transplant setting can be unpredictable since all patients will be immunocompromised to some degree in order to prevent graft rejection. Consequently any study, which investigates the effect of tumour cell growth in a transplant model to investigate whether the immunosuppressive treatment, or MHC mismatch has any bearing on tumour growth is worthwhile.

The stability of the tumour cells (BP36B) used for our study has been demonstrated by the observation that the cells retained their properties after multiple passages(11). The cells being of Wistar origin made it possible for us to study the effects of tissue matching on the tumour behaviour by using outbred Wistar and inbred Lewis strains for implantation. When these cells were injected in the Lewis animals, they behaved as a poorly matched group as the transplantation was between two different strains with marked immunological differences. When these cells were injected into the Wistar rats, they behaved as relatively well-matched combination when compared to the Lewis animals but strictly speaking they could not be classified as syngeneic transplantation. This is because of inter-individual variations in RT1 (rat major histocompatibility complex) among any outbred strain of the rats(15,17,18). This slight variation made our tumour model closely reflective of scenarios in human transplantation; as even the very well matched individuals (excluding identical twins-syngeneic transplantation) would have subtle differences in histocompatibility loci due to the very wide variations in the HLA haplotype(19).

Tumour cells injected into hosts normally take a long time to become palpable. Even cells with short doubling times often take a long time to become clinically significant and enable accurate measurements. The BP36B cells, used in our study took two

months to establish when transplanted in immunocompromised nude mice(11). Consequently we decided to transfect the tumour cells with luciferase in order to detect and monitor tumour growth by sensitive bioluminescent imaging techniques. Furthermore, the quantitative measurements made by this method were objective and less susceptible to human error and bias since tumour load was calculated computationally by signal intensity from the injected tumour cells rather than the more subjective method of visually grading the tumour size.

The behaviour of well and poorly matched transplanted tumour cells under conditions of immunosuppression and rejection (treatment withdrawal) has potential important clinical implications. The tumour cells were, as expected, rejected in the absence of any immunosuppression in both groups of animals since there are likely to be some differences between the donor and recipient even in the well-matched combination. However, the time taken for the poorly matched animals to reject the tumour was significantly shorter (p 0.001), and it is likely that this was due in part to the stronger allogeneic response having an anti-tumour effect. Similar results were noted when the immunosuppression was withdrawn midway in the study period to monitor the effects of rejection (p 0.039). All the Lewis animals rejected the tumour two weeks after withdrawal while only half in the well-matched group did so. The clinical significance of this finding is that if we were to transplant kidneys after *ex vivo* resection of T1a tumours, then perhaps choosing a less well-matched donor recipient combination would be preferable. This would mean, should a recurrence occur in the recipient, simply withdrawing the immunosuppression (with transplant nephrectomy) may aid “rejection” of extra renal tumour cells(20). This was the approach utilised by Nicol et al. in their series, although they were not able to test this hypothesis as the only patient developing recurrence in their series declined any further treatment(7).

The other clinically significant implication is the fact that under standard immunosuppression the tumour continued to grow. There were subtle but statistically significant ($p = 0.033$) differences in the rate of growth, with higher rate of tumour growth in well-matched animals. However, in both strains by the end of study period the signal intensity was high and in the majority of immunosuppressed rats the tumours were palpable. Therefore, the risk of unchecked tumour growth and perhaps metastasis would be a real concern should a tumour be transplanted inadvertently with a restored organ. The behaviour of tumours with immunosuppression using more contemporary immunosuppressants that have reported anti-neoplastic activity, such as Rapamycin and Leflunomide, needs to be investigated. Such immunosuppressive agents may prove to be effective in preventing recurrence or eliminate the cancer cells should they be transplanted inadvertently. A strategy to transplant these kidneys into less well-matched recipients and to use non-calcineurin inhibitor immunosuppression may provide the best outcomes.

6 Conclusions

Subtle variations in the growth of the tumour cells based on MHC-dependent differences in various experimental conditions were detected with great accuracy using the IVIS spectrum imaging system. There are two clinically relevant deductions of our experiments. Firstly, transplanted tumour cells continue to grow unchecked in immunosuppressed hosts. This finding makes it of paramount importance that any kidney transplanted after *ex vivo* resection must be devoid of any tumour load. Secondly, poorly matched combination of donor and hosts were significantly better in rejecting any donor-derived tumour if immunosuppression was withdrawn in this

animal model. *Should a recurrence occur in a clinical situation after such transplants, it might be better to have less well matched donor recipient combination so that host's own immune system can be used at least in part to reject the transplanted tumour by withdrawal of immunosuppression.*

List of Abbreviations

RCC	Renal cell carcinomas
NSS	Nephron sparing surgery
NHEN	N-ethyl-N-hydroxyethylnitrosamine
HLA	Human leukocyte antigen
GFP	Green Florescent Protein
ROIs	Regions of interest
Cyc	Cyclosporine
MHC	Major histocompatibility complex

Competing interests

No author has any competing interest to declare.

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Legends to Figures

Fig 1. IVIS spectrum image of non transfected cells (Left) and transfected cells (right). The system produces a heat map image that can be compared to the scale seen to the right of the image and the intensity of the luminescence calculated (P/sec/cm²/sr).

Fig 2. Day 0 IVIS spectrum image of Wistar rat after injection of transfected tumour cells into the right flank. Imaging was performed 15 minutes after intra-peritoneal injection of Luciferin for maximum signal intensity. Region of interest (ROI, solid red circle) is the area of positive signals from the injection site while the background bioluminescence (dotted red circle) is calculated for each image to calculate bioluminescence.

Fig 3. Graph showing the time taken for rejection of tumour cells among control animals without immunosuppression. Well-matched Wistar animals (green) took two weeks to reject the tumour load while poorly matched Lewis (blue) animals rejected the tumour load within one week ($P < 0.001$) (the error bars representing standard error of mean).

Fig 4. Cyclosporine treatment continue group: Comparison between well matched Wistar (green) and poorly matched Lewis (blue) groups. Transplanted tumour cells continued to grow between both the groups but again the growth was stronger in well-matched animals compared to the poorly matched combination.

Fig 5. Effect of treatment withdrawal in well matched and poorly matched combination groups. All the poorly matched animals (blue) rejected the transplanted

tumour cells within four weeks of study period as opposed to only 50% in well-matched Wistar (green) during the same study period.

Fig 6. Serial IVIS scans of Lewis rats: At the end of 2 weeks of immunosuppression (left), 1 and 2 week post treatment withdrawal (middle and right). Tumour continued to grow when animals were kept on cyclosporine immunosuppression. With treatment withdrawal there has been steady rejection of tumour cells till all the injected cells were destroyed (significantly stronger rejection than well matched combination of Wistar animals).

Fig 1

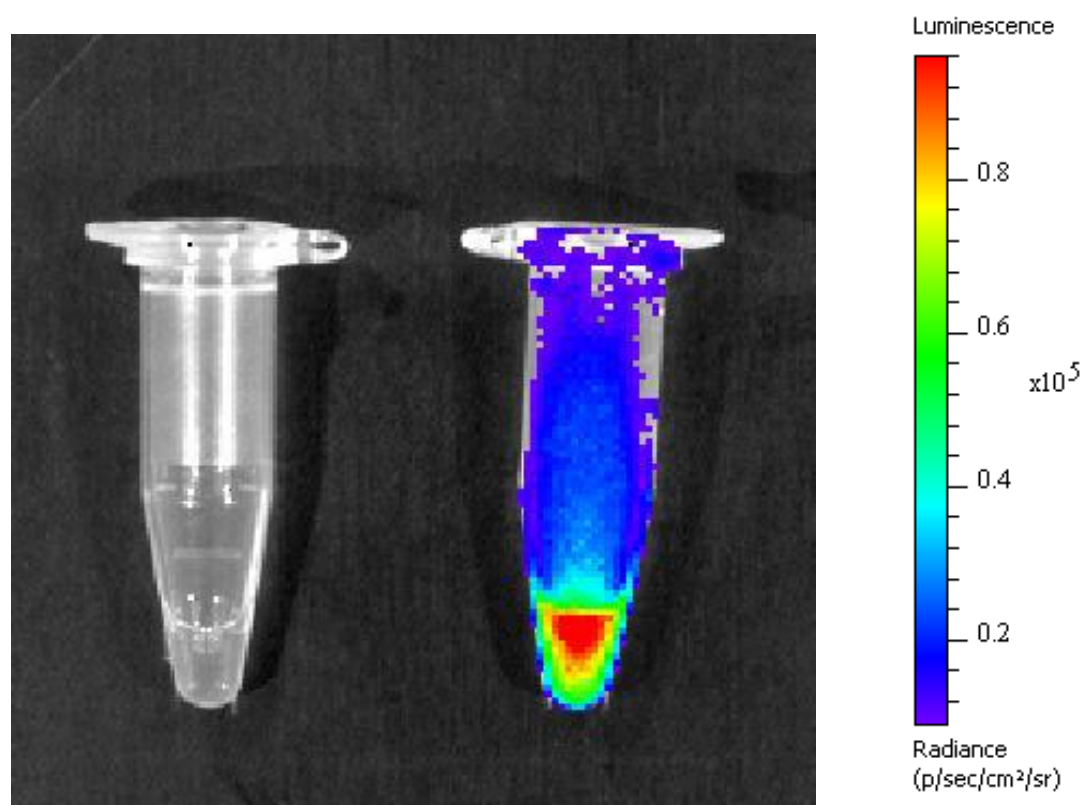


Fig 2

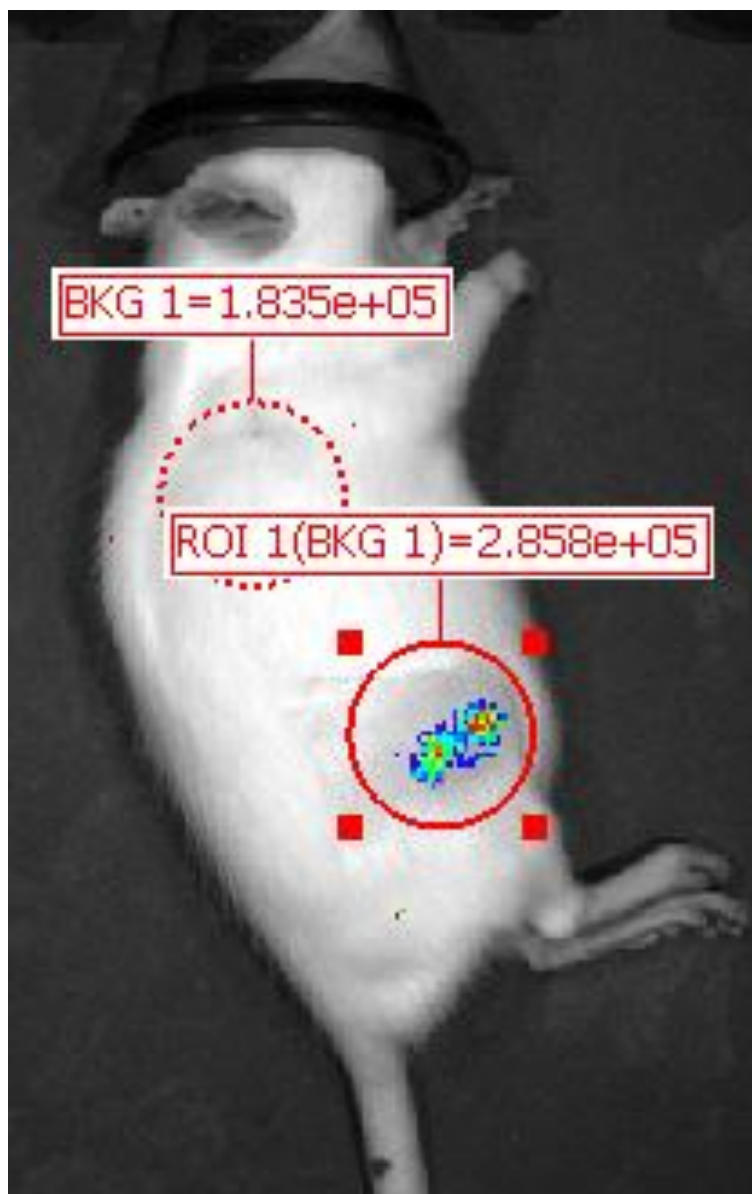


Fig 3

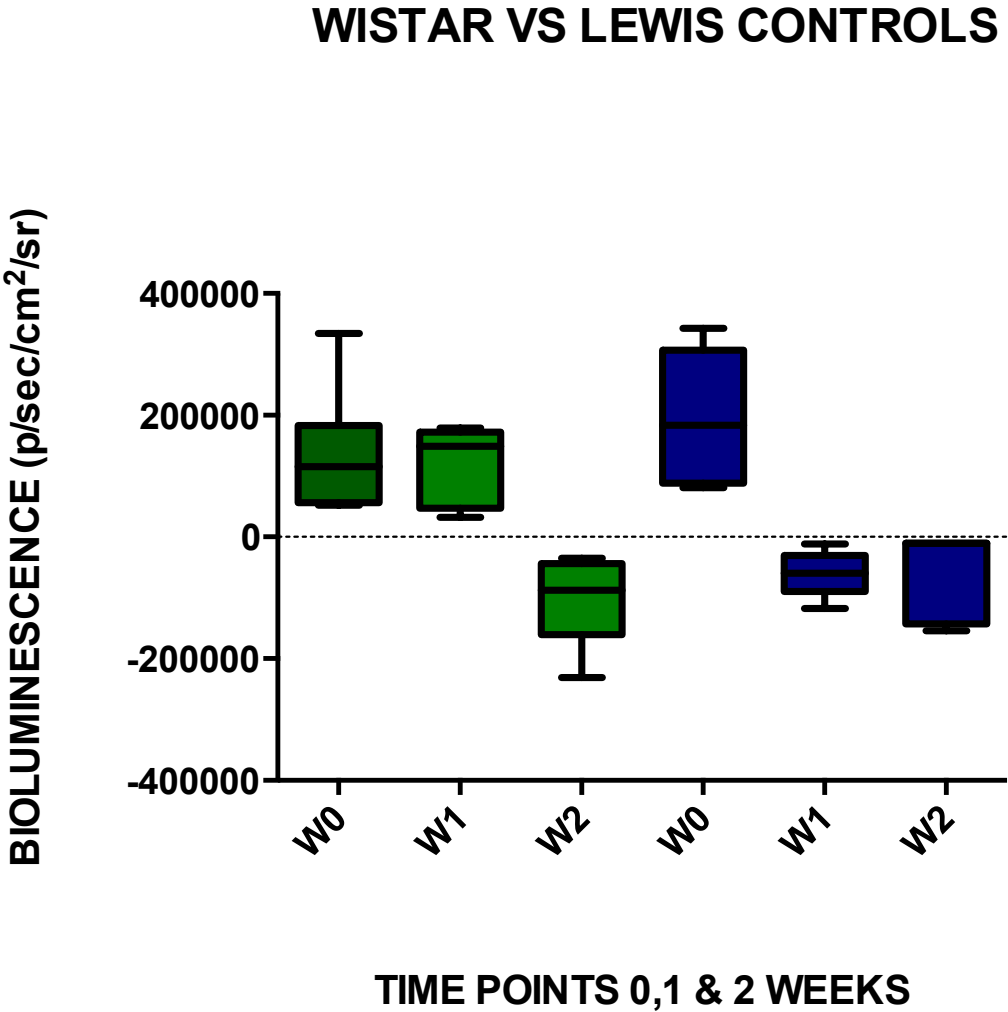


Fig 4

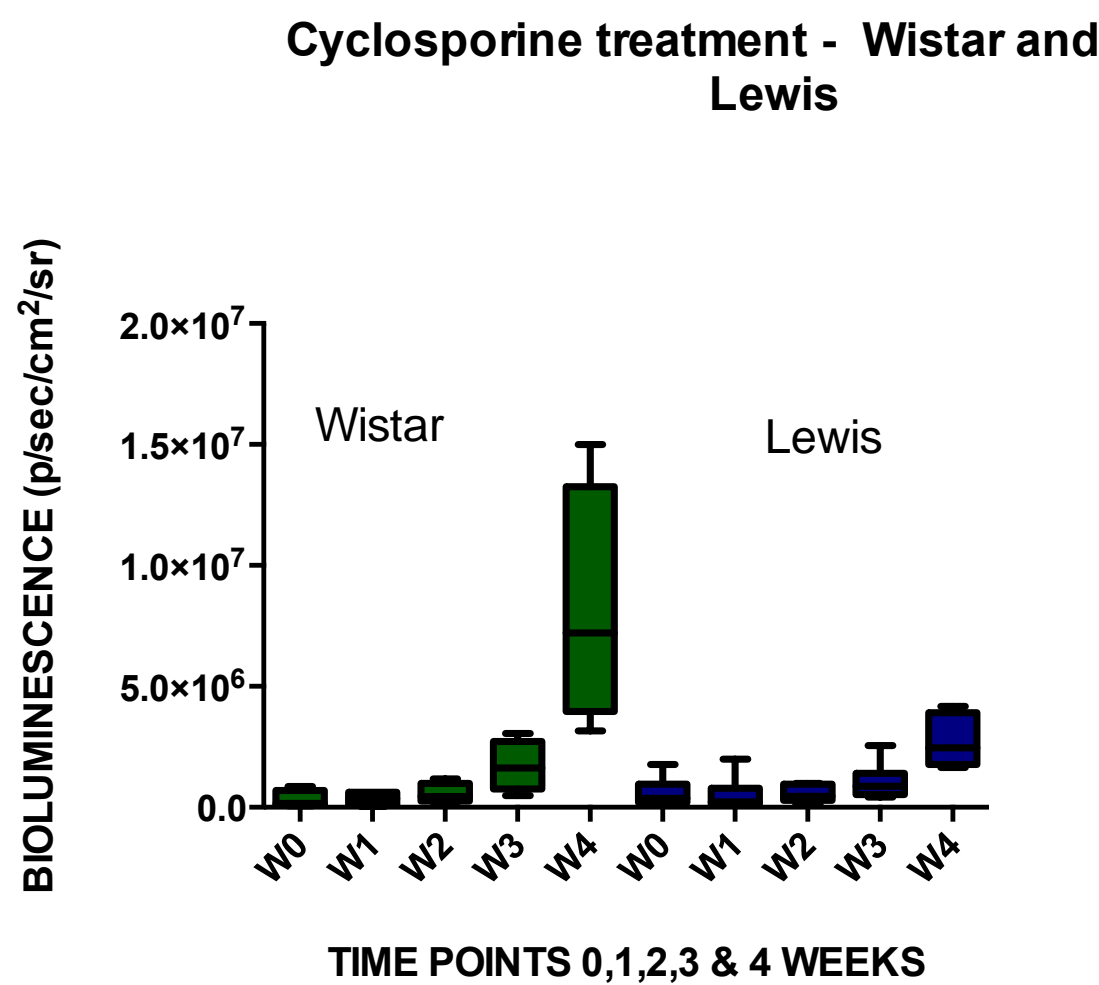


Fig 5

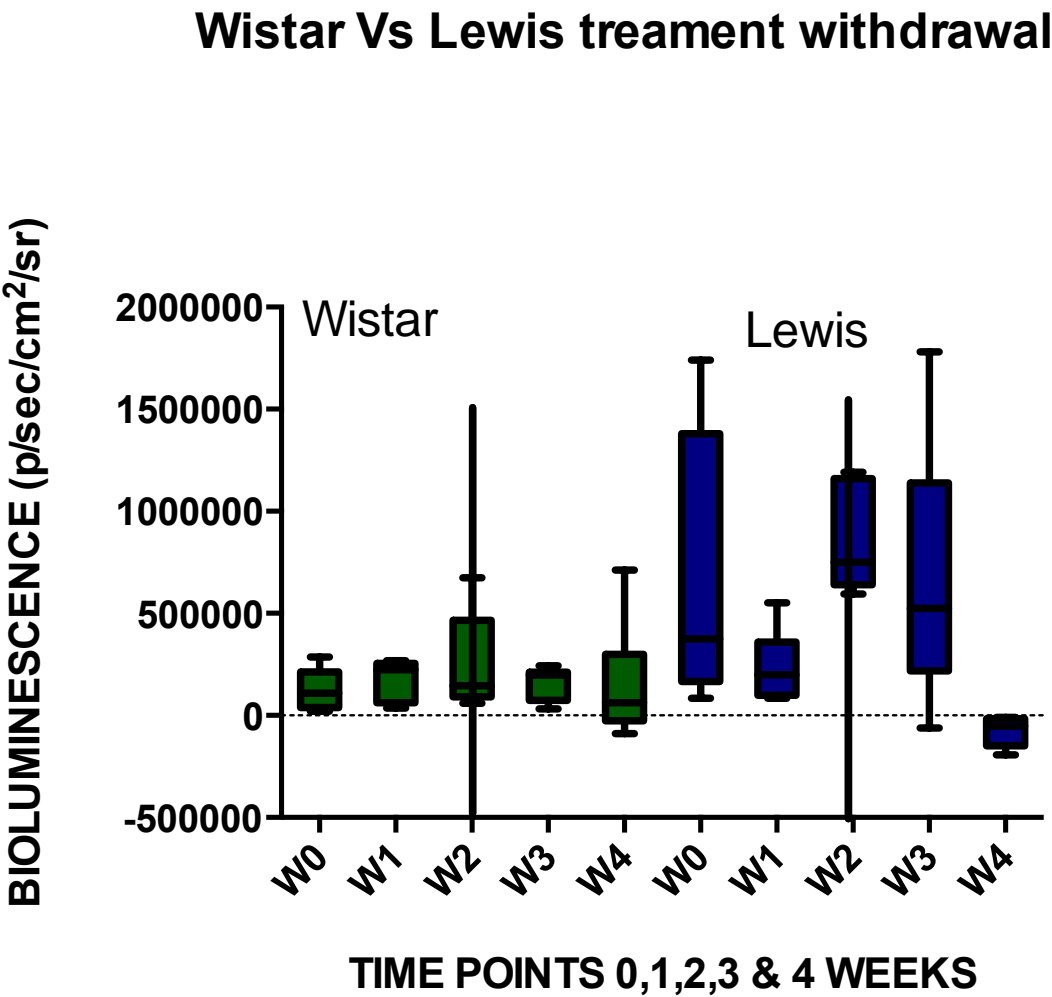
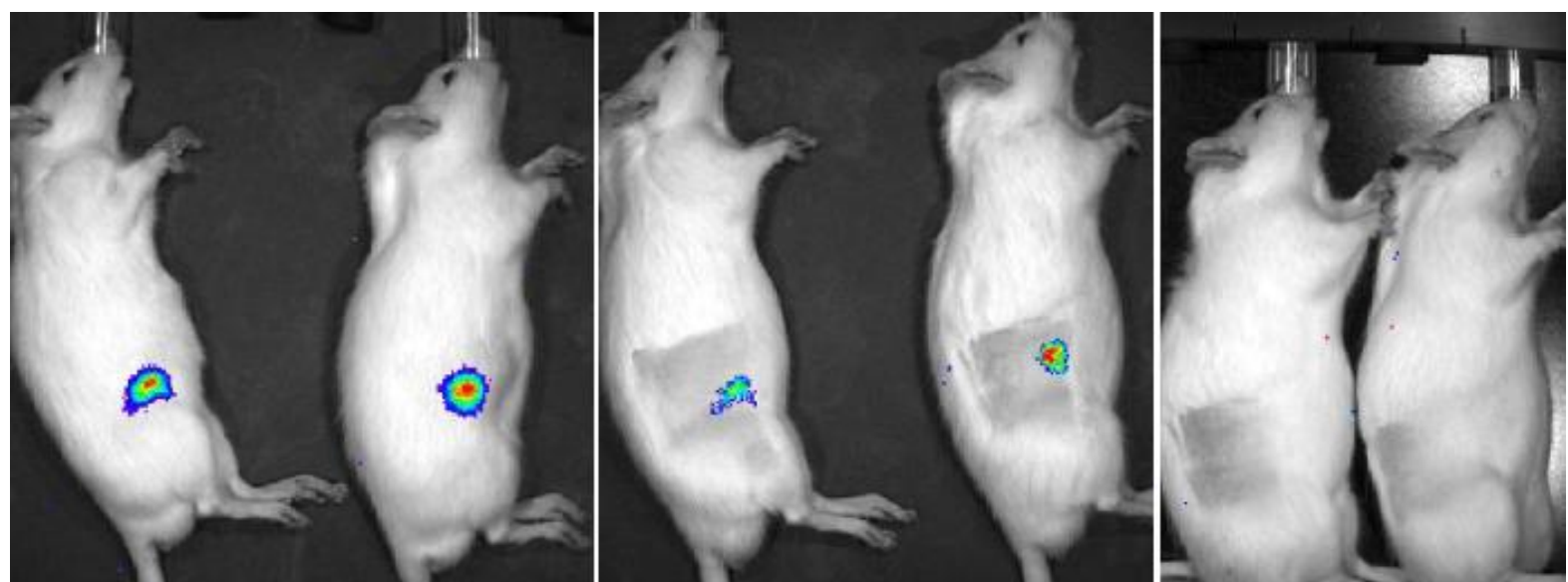


Fig 6



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